Plant Physiol. (1991) 97, 1044–1050
0032-0889/91/97/1044/07/$01.00/0

Received for publication April 30, 1991
Accepted July 2, 1991

Molecular Basis of Imidazolinone Herbicide Resistance in *Arabidopsis thaliana* var Columbia[1]

Kanagasabapathi Sathasivan[2], George W. Haughn, and Norimoto Murai*

Department of Plant Pathology and Crop Physiology, College of Agriculture, Louisiana State University, Baton Rouge, Louisiana 70803 (K.S., N. M.), and Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan S7N OWO, Canada (G.W.H.)

ABSTRACT

Acetolactate synthase (ALS), the first enzyme in the biosynthetic pathway of leucine, isoleucine, and valine, is inhibited by imidazolinone herbicides. To understand the molecular basis of imidazolinone resistance, we isolated the ALS gene from an imazapyr-resistant mutant GH90 of *Arabidopsis thaliana*. DNA sequence analysis of the mutant ALS gene demonstrated a single-point mutation from G to A at nucleotide 1958 of the ALS-coding sequence. This would result in Ser to Asn substitution at residue 653 near the carboxyl terminal of the matured ALS. The mutant ALS gene was introduced into tobacco using *Agrobacterium*-mediated transformation. Imidazolinone-resistant growth of transformed calli and leaves of transgenic plants was 100-fold greater than that of nontransformed control plants. The relative levels of imidazolinone-resistant ALS activity correlated with the amount of herbicide-resistant growth in the leaves of transgenic plants. Southern hybridization analysis confirmed the existence of transferred ALS gene in the transformant showing high imazapyr resistance. The results demonstrate that the mutant ALS gene confers resistance to imidazolinone herbicides. This is the first report, to our knowledge, of the molecular basis of imidazolinone resistance in plants.

Imidazolinones are a group of potent herbicides with long-term broad spectrum activity and low mammalian toxicity (16). Imidazolinones are comprised of the nonselective herbicide imazapyr and the selective herbicides, imazaquin and imazethapyr. Several lines of evidence suggest that the primary target site of imidazolinones is the enzyme ALS[3] (EC 4.1.3.18), which catalyzes the first common step in the biosynthetic pathway of leucine, isoleucine, and valine. Imidazolinone treatment in maize tissue suspension culture resulted in reduced biosynthesis of the branched chain amino acids, and the imidazolinone toxicity could be reversed by exogenous supply of the branched chain amino acids (25). Imidazolinones inhibit ALS activity in *in vitro* enzyme assays (24). Finally, many mutant plants resistant to imidazolinones also have imidazolinone-resistant ALS activity [*Zea mays* (25), *Datura innoxia* (21), *Chlamydomonas reinhardtii* (30), and *Arabidopsis thaliana* (7)].

In addition to imidazolinones, ALS is also inhibited by a structurally diverse group of herbicides *viz.* sulfonylureas (2, 17), triazolopyrimidines (3), and pyrimidyl-oxy-benzoate (8, 28). Schloss *et al.* (23) proposed that sulfonylureas, imidazolinones, and triazolopyrimidines compete for a common binding site on a bacterial ALS based on herbicide-enzyme-binding studies. However, several spontaneous and chemically induced mutant plants and their enzymes showed varied levels of cross-resistance to these ALS inhibitors (4, 6, 19, 21, 28, 30), suggesting that these herbicides may interact with non-overlapping binding sites on ALS.

We were interested in determining the molecular basis of imidazolinone resistance for four reasons. First, such information will enhance our understanding of the interaction between imidazolinones and ALS. Second, it might elucidate the causes for lack of cross-resistance in many mutant plants to both imidazolinones and sulfonylureas, indicating that their sites of interaction on ALS may be different. Third, it will provide an explanation as to how weeds develop resistance to imidazolinones (4, 19) and suggest a basis for developing sound weed control programs. Finally, isolation of a plant ALS gene conferring imidazolinone resistance will permit the introduction of an imidazolinone resistance trait to crop varieties and provide a useful selectable marker in plant transformation.

A homozygous mutant line (GH90) of *Arabidopsis* has been isolated and characterized by Haughn and Somerville (7). GH90 is approximately 100-fold more resistant to imazapyr than the wild-type plants due to imazapyr-resistant ALS activity. Genetic analysis indicated that the imidazolinone resistance is due to a single dominant nuclear mutation, *imr*, closely linked to the ALS locus. The mutant GH90, however, is not resistant to sulfonylureas (7) and triazolopyrimidines (K. Sathasivan and N. Murai, unpublished results). We isolated the ALS gene from the mutant GH90 after screening a genomic DNA library with an ALS probe from pGH1 (20). The ALS gene in a 5.8-kb *Xba*I fragment was subcloned into pUC18. We sequenced both strands of this ALS gene and identified a single-point mutation from G to A at nucleotide 1958 of the coding sequence. This mutation predicted the substitution from Ser to Asn at residue 653 of ALS. In this report, we provide evidence that the isolated mutant ALS gene confers imazapyr resistance to transgenic tobacco plants.

[1] This work was supported in part by research assistantship to K.S. provided by the Louisiana State University College of Agriculture and Dr. John B. Baker and by grants from Louisiana Education Quality Support Funds (1987–90)-RD-A-6 to NM.

[2] Present address: Department of Botany, The University of Texas at Austin, Austin, TX 78713.

[3] Abbreviations: ALS, acetolactate synthase; kb, kilobase.

Our results demonstrated that the single amino acid substitution from Ser to Asn near the carboxyl terminus of ALS is the molecular basis for imazapyr resistance in the GH90 mutant.

MATERIALS AND METHODS

Plants, Bacterial Strains, and Plasmids

Seeds of both wild-type and mutant GH90 (*csr1-2/csr1-2*, [7]) *Arabidopsis thaliana* var Columbia were germinated and grown on an autoclaved perlite/vermiculite/spaghnum mixture (1:1:1) as described previously (6). *Escherichia coli* strain XL1-Blue and the phagemid pBluescript were obtained from Stratagene and used for all vector construction and subcloning. *Agrobacterium tumefaciens* strain LBA4404 containing the disarmed Ti plasmid was used for tobacco transformation. Plasmid pGH1 contains an ALS gene encoding for sulfonylurea resistance as described by Haughn *et al.* (5). Plasmid pKS1 contains the ALS gene from GH90 in the 5.8-kb *Xba*I fragment isolated from the λ clone Imr3ALS3 (20). Plant transformation vector pTRA409 is a 11.5-kb broad-host range plasmid containing the right and left transferred-DNA border of Ti plasmid flanking the neomycin phosphotransferase II (*NPTII*) gene under control of the promoter and terminator of a tumor morphology large (*tml*) gene of *A. tumefaciens*. pTRA409 has a prokaryotic tetracycline resistance gene and a unique *Xba*I site.

Chemicals

Imazapyr and imazaquin were obtained from American Cyanamid Co., Princeton, NJ. Triazolopyrimidine was a gift from Dr. M. Subramanian, Dow-Elanco Chemical Co., Walnut Creek, CA.

Nucleic Acid Techniques

Standard techniques of Maniatis *et al.* (11) were followed. The 3′ regions of the wild-type and mutant ALS gene were subcloned into pBluescript at the *Bgl*II to *Nsi*I restriction site, and the plasmid DNA was sequenced with Sequenase (US Biochemical) by the chain termination method of Sanger *et al.* (18). Total DNA from tobacco leaves was isolated as described by Nagao *et al.* (14). Total *Arabidopsis* DNA was isolated as described by Leutwiler *et al.* (10). DNA from the nontransformed and transgenic plant X435-3A was digested with *Eco*RI, fractionated on a 0.8% (w/v) agarose gel, transferred to nitrocellulose membrane (Schleicher and Schuell), and hybridized with a 2.1-kb *Eco*RI probe containing the 5′ region of the ALS gene.

Construction of Plant Transformation Vector

A 4.6-kb *Cla*I to *Xba*I fragment from pKS1 containing the 2.0-kb ALS-coding, 1.25-kb 5′-upstream, and 1.35-kb 3′-downstream regions was subcloned into pBluescript to create pBKS1. This placed a *Kpn*I site 5′ adjacent to the *Cla*I site. A 1.2-kb *Eco*RI fragment containing a Tn903-derived neomycin phosphotransferase gene was subcloned into pBluescript to generate pBKan from which the kanamycin marker was isolated as the 1.2-kb *Kpn*I to *Xba*I fragment. A 4.6-kb *Kpn*I to *Xba*I fragment from pBKS1 containing the ALS gene was ligated with the 1.2-kb *Kpn*I to *Xba*I from pBKan. The resulting 5.8-kb *Xba*I fragment was cloned into a *Xba*I site of pTRA409 to generate the plant expression vector pTRA435. The kanamycin marker facilitated the subcloning of ALS gene into the 11.5-kb pTRA409 vector. Plasmid pTRA435 containing the mutant ALS gene has been transformed to *A. tumefaciens* by a freeze-thaw method (1).

Tobacco Transformation

Details of leaf disc cocultivation, media composition, and the initial selection procedure have been described by Burow *et al.* (1). Leaf discs from *Nicotiana tabacum* cv Xanthi were cocultivated with *A. tumefaciens* containing pTRA435. As controls, tobacco leaf discs were also cocultivated with *A. tumefaciens* containing pTRA409 only, and plants were regenerated in a similar fashion. Cocultivated leaf discs were transferred to callus media containing 300 mg/L kanamycin and 500 mg/L carbenicillin and maintained for 3 weeks. Calli from independent leaf discs were transferred to shooting media containing 100 mg/L kanamycin and 500 mg/L carbenicillin. Shoots and their corresponding calli were transferred to rooting and callus maintenance media, respectively, containing 100 mg/L kanamycin. Nontransformed calli and their shoots were maintained in appropriate media without kanamycin. Rooted plantlets were transferred to soil and grown to maturity in greenhouse. Seeds were collected from mature plants for genetic analysis.

Evaluation of Herbicide Resistance

Approximately 10-d-old calli were placed in callus maintenance media containing various concentrations of imazapyr. All plates contained 500 mg/L carbenicillin in addition to the herbicides to suppress the growth of residual *A. tumefaciens*. Imazapyr was dissolved in sterile 10 mM potassium phosphate buffer, pH 7.5, to prepare 50 mM stock solution, and the stock was diluted to obtain 1 mM, 100 μM, 10 μM, 1 μM, 100 nM, and 10 nM final concentrations. Each treatment was replicated four times, and the experiments were conducted at least three times. Callus fresh weight was taken after 21 d of incubation at 25°C under continuous fluorescent light (56 $\mu E\ m^{-2}\ s^{-1}$).

For leaf cutting assay, healthy young leaves from regenerated plants were soaked for sterilization in a solution of 40% (v/v) bleach and 0.02% Triton X-100 (v/v) and rinsed with sterile water. Sterilized leaves were cut into approximately 0.25-cm squares and placed in callus maintenance media containing various concentrations of herbicide. The experiment was conducted twice and replicated five times per treatment. After 21 d of incubation at 25°C under continuous fluorescent light, the leaf cuttings were photographed.

ALS Assay

Activity of ALS was measured based on the method described by Chaleff and Mauvais (2) and modified by Haughn and Somerville (6) and Singh *et al.* (26). Fresh leaf or calli

samples (3 g) were ground to a fine powder in liquid N_2. The powder was mixed in 10 mL cold extraction buffer containing 100 mM potassium phosphate, pH 7.5, 10 mM $MgCl_2$, 40 mM sodium pyruvate, 500 μM thiamine pyrophosphate, 23 μM flavin adenine dinucleotide, and 1% (w/v) PVP. The homogenate was passed through cheesecloth and centrifuged at 27,000*g* at 4°C for 10 min, and the supernatant was aliquoted into 200-μL volumes in Eppendorf tubes. Tenfold herbicide concentrations (50 μL) and twofold assay buffer (250 μL 65 mM potassium phosphate, pH 7.5, 10 mM $MgCl_2$, 100 mM sodium pyruvate, 250 μM thiamine pyrophosphate, and 23 μM flavin adenine dinucleotide) were added to each aliquot. The reaction mixtures were incubated at 37°C for 90 min. The conversion of acetolactate to acetoin and the measurement of acetoin produced were as described before (2).

RESULTS

Single-Point Mutation Near the 3′ End of ALS Gene

The cloning and entire DNA sequence of the mutant ALS gene from GH90 has been reported previously (20). A single-point mutation from G to A was identified at the nucleotide 1958 of the ALS-coding region. The restriction map of a part of the λ clone Imr3ALS3 containing the ALS gene is shown in Figure 1A. To confirm the mutation reported earlier, the 3′ region of the mutant and wild-type ALS gene was subcloned into pBluescript and the plasmid DNA was sequenced as shown in Figure 2A. A mutation from guanosine to adenosine was observed at the nucleotide 1958 near the 3′ end of the ALS-coding sequence. This would result in Ser to Asn substitution at residue 653 of the matured protein as shown in Figure 2B. This ALS mutation in the imazapyr-resistant

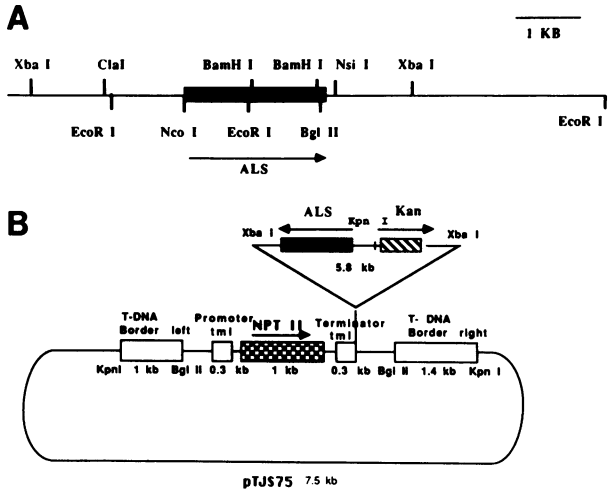

Figure 1. A, Restriction endonuclease maps of the λ clone Imr3ALS3; B, physical organization of the plant transformation vector pTRA435 containing the ALS gene from *A. thaliana* mutant GH90. pTRA435 consists of a broad-host range plasmid pTJS75, transferred-DNA (T-DNA) right and left borders, a plant-expressible kanamycin-resistance gene (NPTII), a Tn903-derived neomycin phosphotransferase gene (kan), and the mutant ALS gene containing the 2.0-kb coding, 1.25-kb 5′-upstream, and 1.35-kb 3′-downstream regions.

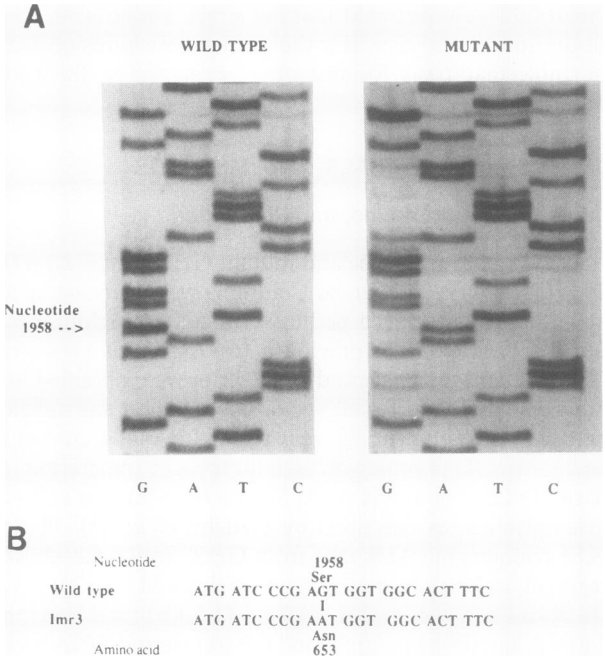

Figure 2. A single-point mutation in the ALS gene from the imidazolinone-resistant GH90 mutant. A, The *Bgl*II to *Nsi*I fragment from the 3′ region of the ALS-coding sequence was subcloned into pBluescript and the sequence was determined by the double-stranded DNA-sequencing technique after fractionation on a 6% (w/v) polyacrylamide gel. B, Comparison of the wild-type and mutant Imr-3 sequences.

GH90 is unique in its position closest to the carboxyl terminus, compared to the ALS mutations conferring sulfonylurea resistance in higher plants (9, 13) as summarized in Table I. The mutation occurs at Ser residue 653 which is conserved among the known plant species but not among yeast and procaryotes (12, 29) as shown in Figure 3, implying the importance of this region to plant ALS.

Expression of Herbicide Resistance in Transgenic Tobacco

The ALS gene from GH90 was used to transform the *N. tabacum* cv Xanthi to test its ability to confer the imidazolinone-resistant phenotype. A 4.6-kb *Cla*I to *Xba*I fragment containing the 2.0-kb ALS-coding, 1.25-kb 5′-upstream, and 1.35-kb 3′-downstream regions was subcloned along with a 1.2-kb prokaryotic kanamycin marker gene into a 11.5-kb plant transformation vector pTRA409 to generate pTRA435 as shown in Figure 1B. pTRA435 was introduced into *A. tumefaciens* by a freeze-thaw method. Tobacco leaf discs were transformed after cocultivation with *A. tumefaciens*, and transformants were selected in the presence of 300 mg/L kanamycin (1). Transformed calli from independent leaf discs were transferred to shooting media containing 100 mg/L kanamycin for secondary selection. Regenerated shoots and the corresponding calli were transferred to rooting media and callus maintenance media, respectively, containing 100 mg/L kanamycin. Transformed calli were initially tested for her-

Table I. *Mutations in ALS Conferring Herbicide Resistance in Plants*

Species and Mutant	Amino Acid	Mutation			Resistance to	Reference
A. thaliana						
GH 50	197	CCT Pro	→	TCT Ser	Sulfonylurea	(5)
GH 90	653	AGT Ser	→	AAT Asn	Imidazolinone	This paper
N. tabacum						
SuRA-C3	196	CCA Pro	→	CAA Gln	Sulfonylurea	(9)
SuRB-S4HRA	196 and 573	CCA Pro TGG Trp	→	GCA Ala TTG Leu	Sulfonylurea and imidazolinones	(9, 13)

bicide resistance even though they represented a mixture of independent transformants. Because the primary and secondary selections for the transformants were based on the cotransformed kanamycin resistance and not based on imidazolinone resistance, it precluded the possibility of picking up spontaneous mutants resistant to the herbicide.

Calli derived from independently transformed leaf discs X435-6A, -12A, and -13 were plated on callus maintenance media containing different concentrations of imazapyr. As shown in Figure 4, nontransformed control calli grew in imazapyr concentrations up to 100 nM. Transformed calli X435-13, -12A, and -6A grew on media containing up to 1, 10, and 100 μM imazapyr, respectively, showing 10-, 100-, and 1000-fold resistance to the nonselective herbicide imazapyr. The imazapyr-resistant growth of the transformed calli is due to the introduced mutant ALS gene rather than a higher growth rate, because the growth of nontransformed calli was greater than that of the transformants in the absence of herbicide. The difference in the levels of imazapyr resistance among the transformed calli may reflect copy number difference or differential expression of the ALS gene due to the chromosomal position effects in the tobacco genome.

Imazapyr Resistance in Leaves of Transgenic Plants

Because the primary calli tested may be a mixture of several independent transformants, leaves of the regenerated plants were assayed to confirm the herbicide resistance of the cell population having a homogeneous genetic background. Similar tests using leaf cuttings to assay for herbicide resistance have been described by Stalker *et al.* (27) and Olszewski *et al.* (15). Leaf cuttings of transformed and nontransformed plants were sterilized and placed on callus maintenance media containing various concentrations of the herbicide. As shown in Figure 5 (section 1), nontransformed control leaf induced callus growth up to the imazapyr concentration of 100 nM. Leaf cuttings of transgenic plants X435-3A (Fig. 5, section 2) grew up to 10 μM imazapyr and demonstrated the highest 100-fold resistance. Leaves of transformed plants X435-8A and -8D (Fig. 5, sections 4 and 5) showed 10-fold resistance and grew in the presence of up to 1 μM imazapyr. Transformants X435-6B and -12A (Fig. 5, sections 3 and 8) grew at the <1 μM herbicide concentration. It should be mentioned that the sterilization treatment of leaf with 40% (v/v) bleach and 0.02% (v/v) Triton X-100 appeared to increase the susceptibility to the herbicide, resulting in detrimental effects in the

Arabidopsis Wild Type:	E	H	V	L	P	M	I	P	S	G	G	T	F	N	D	V
GH90:	E	H	V	L	P	M	I	P	N	G	G	T	F	N	D	V
Tobacco:	E	H	V	L	P	M	I	P	S	G	G	A	F	K	D	V
Brassica:	D	H	V	L	P	L	I	P	S	G	G	T	F	K	D	I
Yeast:	V	P	V	L	P	M	V	A	G	G	S	G	L	D	E	F
E. coli ALS1:	E	K	V	Y	P	M	V	P	P	G	A	A	N	T	E	N
ALS2:	E	N	V	W	P	L	V	P	P	G	A	S	N	S	E	M
ALS3:	S	T	S	T	R	C	R	F	A	G	A	E	W	M	K	C

Figure 3. Conservation of deduced amino acid residues in the ALS near the mutation site conferring imidazolinone resistance. The nucleotide sequences were obtained from Mazur *et al.* (12) and Wiersma *et al.* (29).

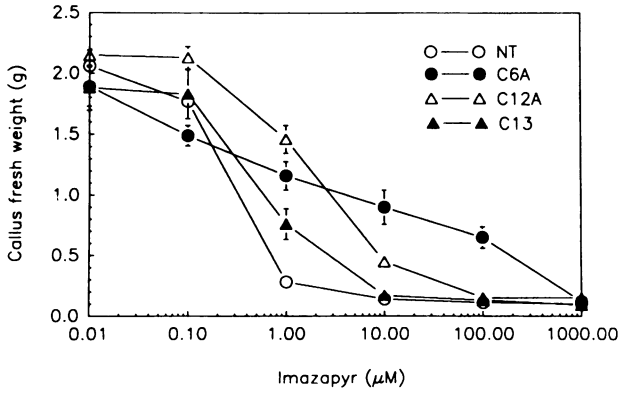

Figure 4. Imazapyr-resistant growth of transformed tobacco calli. Calli derived from independently transformed leaf discs X435-6A, -12A, and -13 were grown in various concentrations of imazapyr for 21 d, and the fresh weight of calli was determined. Experiments were conducted three times and each data point represents an average of four replications. NT, nontransformed control calli; C6A, C12A, and C13, transformed calli derived from independent leaf discs X435-6A, -12A, and -13, respectively.

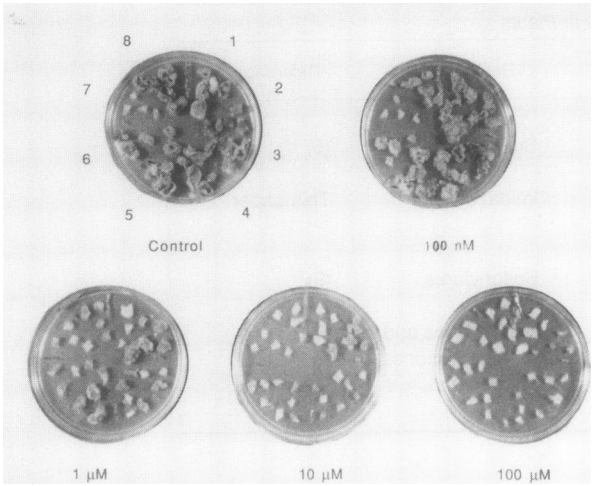

Figure 5. Expression of imazapyr-resistant phenotype in leaves of regenerated tobacco plants. Leaf cuttings of nontransformed (section 1) and transformants X435-3A (section 2), X435-6B (section 3), X435-8A (section 4), X435-8D (section 5), X435-9B (section 6), X435-11A (section 7), and X435-12A (section 8) were grown on callus maintenance media containing various imazapyr concentrations for 21 d. Experiments were conducted twice. Representative plates of five replications are shown.

case of X435-11A (Fig. 5, section 7). Similar variations in the herbicide resistance levels were observed by Stalker *et al.* (27) in tobacco plants transformed with the bromoxynil detoxification gene. The levels of imazapyr-resistant growth in the transformants X435-3A and -8A showing 100- and 10-fold resistance, respectively, were well correlated with the levels of herbicide-resistant enzyme activity shown below.

Expression of Imazapyr-Resistant ALS Enzyme Activity

ALS activity was assayed in leaves of nontransformed and transformed plants to demonstrate that the imazapyr-resistant phenotype is due to the altered enzyme activity. Crude extracts were prepared from young leaves and directly used for herbicide inhibition studies using imazapyr concentrations from 100 nM to 1 mM. Figure 6 shows the uninhibited ALS activity at each herbicide concentration plotted as a percentage of the control ALS activity in the absence of herbicide. The ALS activity in leaves of nontransformed plant (NT) and vector-transformed plants (X409-H1) was greatly inhibited at the concentration of 10 μM imazapyr. In contrast, >50% of ALS activity in transformants X435-3A and -8A was resistant to the same concentration of imazapyr. The resistant ALS activity in these transformants remained even up to a concentration of 1 mM imazapyr, showing a 1000-fold resistance. A fraction of ALS activity in transformants was susceptible to imazapyr because the transformed plants contain the endogenous tobacco ALS genes encoding for imazapyr-sensitive ALS. The higher resistant ALS activity in transformant X435-3A than in transformant X435-8A correlates well with the herbicide-resistant growth in leaf cutting assay. These results indicated that the imazapyr resistance phenotype in the transgenic plants was due to the introduced mutant ALS gene coding for imazapyr-resistant ALS activity.

Southern Analysis

Southern hybridization analysis confirmed the existence of the introduced *Arabidopsis* gene in transgenic tobacco plant X435-3A having the maximum level of imazapyr resistance as shown in Figure 7. Plant genomic DNA digested with *Eco*RI was hybridized to a 2.1-kb *Eco*RI probe, which includes the 0.9-kb ALS-coding and 1.2-kb upstream regions of ALS gene. The expected 2.1-kb band was detected only in DNA from *Arabidopsis* (lane 3) and from the transformed tobacco X435-3A (lane 1). Additional bands of 3.2 and 5.4 kb were observed in transformants X435-3A, probably due to multiple integration of the transferred ALS gene in the tobacco genome. The nontransformant (lane 2) showed a faint 3.2-kb band which could be due to cross-hybridization with endogenous tobacco ALS genes, because the *Arabidopsis* ALS gene shares about 80% sequence similarity to the tobacco ALS gene (13).

DISCUSSION

We demonstrated here that the mutant ALS gene from GH90 confers the imazapyr resistance phenotype in transformed plants. The ALS gene contained a point mutation from G to A at nucleotide 1958 of the coding sequence that predicted a substitution from Ser to Asn at residue 653 of the ALS. This mutant ALS gene transformed imidazolinone-sensitive tobacco into an imidazolinone-resistant plant and encodes an imazapyr-resistant ALS activity. We believe that the imazapyr-resistant ALS activity is due to the single amino acid substitution identified at residue 653. This finding has been independently confirmed by P. Stougaard, Copenhagen, Denmark (personal communication), who introduced this mutation into the sugar beet ALS gene and showed the mutant gene conferring imazethapyr resistance to *E. coli*. If there are additional mutations in the ALS gene (for example, a new *Cla*I site located 1.2-kb upstream of the coding sequence) and

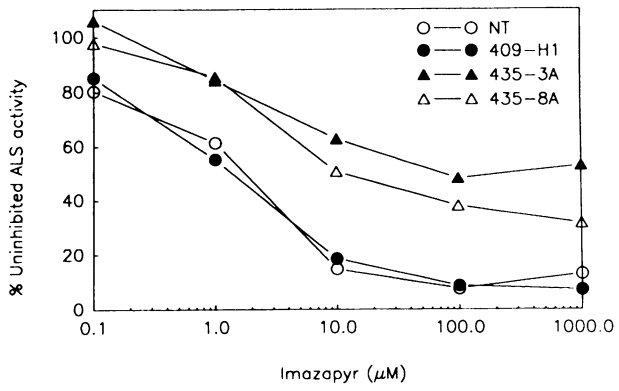

Figure 6. Imazapyr-resistant activity of ALS in crude extracts from leaves of transgenic tobacco plants. The ALS activity was expressed as a percentage of control without any herbicide in crude leaf extracts from the nontransformed (NT), pTRA409-transformed plants (X409-H1), or pTRA435-transformed plants (X435-3A and X435-8A).

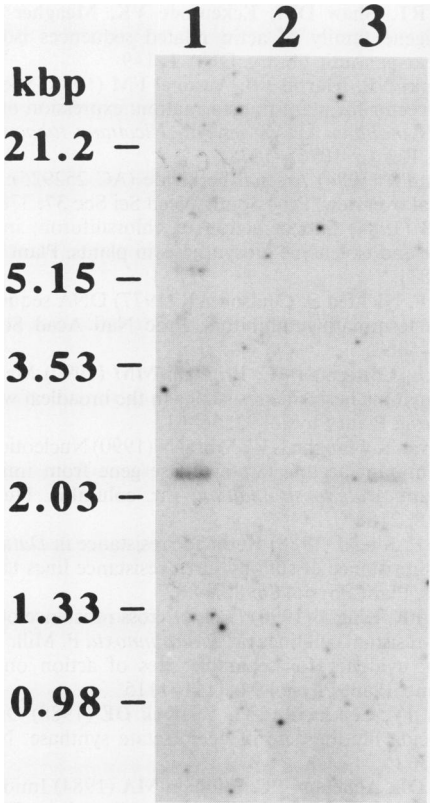

Figure 7. Southern hybridization analysis of DNA from the transformant showing maximum resistance to imazapyr. Total DNA from the transformant X435-3A (lane 1), nontransformed tobacco (lane 2), and wild-type *Arabidopsis* (lane 3) were digested with *Eco*RI and fractionated on a 0.8% (w/v) agarose gel, transferred to nitrocellulose filter, and hybridized to an *Eco*RI 2.1-kb ALS probe from pKS1.

any other open reading frames in the flanking regions of the introduced ALS gene, their importance is not known. However, based on the current evidence of the Asn-653 mutation in ALS showing imazapyr-resistant ALS activity in the transgenic plants, this unique mutation is the molecular basis of imidazolinone resistance in *A. thaliana*. Because the *imr* gene from GH90 (7) probably represents a new imazapyr-resistance allele of the *CSR1* gene of *A. thaliana*, we propose to rename *imr csr1-2*.

Probable Effects of Point Mutations

The mutations listed in Table I might cause an alteration in the herbicide-binding sites on ALS directly or indirectly through changes in the secondary structure of the protein. A structural preference and hydropathy analysis of these mutations (data not shown) in plant ALS listed in Table I indicated that all the mentioned amino acid substitutions are probably on the external surface of the ALS molecule. These data, combined with the normal enzymatic activity of the mutant ALS, suggest that the *csr1-2* mutation does not cause a significant change in the secondary or tertiary structure of ALS. The most probable effect of these mutations is that they cause a minor alteration in electrical charge or stearic hindrance at the herbicide-binding site of the ALS molecule.

The above discussion might also explain the probable causes for the lack of cross-resistance to other ALS inhibitors. Haughn and Somerville (6, 7) showed that the *Arabidopsis* mutants resistant to chlorsulfuron are not cross-resistant to imazapyr and *vice versa*. Saxena and King (21, 22) showed similar results of lack of cross-resistance to both sulfonylureas and imidazolinones in *Datura innoxia* cell lines. In addition, Subramanian *et al.* (28) reported spontaneous mutants of tobacco and cotton cell lines, showing varied levels of cross-resistance to four different types of ALS-inhibiting herbicides and suggested nonoverlapping binding domains for these structurally diverse ALS inhibitors. Here, we show the first molecular evidence that the mutation that did not induce cross-resistance in *Arabidopsis* resides in the distinctly different part of the ALS-coding sequence.

However, we should not conclude, based on this mutation analysis, that the binding domains of imidazolinones and sulfonylureas are different, because two amino acids located far apart in a coding sequence might still fold together to form a single herbicide-binding domain as suggested by Schloss *et al.* (23). The amino acid residues specifically interacting with imidazolinones and sulfonylureas may be different and yet they may constitute the same herbicide-binding domain. Further conclusions concerning the binding sites of different herbicides must await more detailed studies of the three-dimensional structure of the ALS protein.

Herbicide Resistance in Weeds

Hall and Devine (4) observed cross-resistance of the sulfonylurea-resistant biotype of *Stellaria media* to triazolopyrimidines and not to imidazolinones, suggesting that sulfonylurea and triazolopyrimidine may share a common binding site which may be different from that of imidazolinone. The results from the current studies and from Mazur and Falco (13) showed that resistance to sulfonylureas and imidazolinones are caused by different mutations. This implies that the frequency of weeds developing resistance to both of these herbicides (*i.e.* double-mutation frequency) is lower than the chances of developing resistance to only one group (*i.e.* single-mutation frequency). However, considering the large population of weed seeds present in the soil, the probability of developing resistance to more than one ALS-inhibiting herbicides over several cropping seasons is high. Hence, we suggest that herbicides with a target site other than ALS should be rotated or used in combination with these ALS inhibitors.

Other Implications

This mutant ALS gene could be introduced into other crop species to develop imidazolinone-resistant crop varieties. Also, similar mutations could be introduced at the corresponding nucleotide positions of other ALS genes to confer imidazolinone resistance as shown by P. Stougaard (personal communication). This mutant gene could also be used as a selection marker in plant transformation. Moreover, this gene could be expressed in prokaryotic organisms after site-directed mutagenesis to study the mode of action of herbicide inhibition.

ACKNOWLEDGMENTS

We sincerely thank Dr. Chris R. Somerville, Michigan State University, for providing the imazapyr-resistant mutant GH90 of *A. thaliana* and the plasmid pGH1. We are grateful to Drs. Sue G. Bartlett, Simon H. Chang, James V. Moroney, John B. Baker, and Marc A. Cohn for critical reading of the manuscript and to all members of the Plant Molecular Biology Laboratory for discussion and encouragement.

LITERATURE CITED

1. **Burow MB, Chlan CA, Sen P, Lisca A, Murai N** (1990) High frequency generation of transgenic tobacco plants after modified leaf disc cocultivation with *Agrobacterium tumefaciens*. Plant Mol Biol Rep **8:** 124–139
2. **Chaleff RS, Mauvais CJ** (1984) Acetolactate synthase is the site of action of two sulfonylurea herbicides in higher plants. Science **224:** 1443–1445
3. **Gerwick BC, Loney V, Subramanian M** (1987) Mechanism of action of 1,2,4-triazolo [1,5A] pyrimidines. Presented at the American Chemical Society 194th National Meeting, New Orleans, LA, Abstract No. 163
4. **Hall LM, Devine MD** (1990) Cross resistance of a chlorsulfuron-resistant biotype of *Stellaria media* to a triazolopyrimidine herbicide. Plant Physiol **93:** 962–966
5. **Haughn GW, Smith J, Mazur B, Somerville CR** (1988) Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. Mol Gen Genet **211:** 266–271
6. **Haughn GW, Somerville CR** (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol Gen Genet **204:** 430–434
7. **Haughn GW, Somerville CR** (1990) A mutation causing imidazolinone resistance maps to *csr1* locus in *Arabidopsis thaliana* var Columbia. Plant Physiol **92:** 1081–1085
8. **Hawkes TR** (1989) Studies of herbicides which inhibit branched chain amino acid biosynthesis. *In* LG Copping, J Dalziel, AD Dodge, eds, Prospects for Amino Acid Biosynthesis Inhibitors in Crop Protection and Pharmaceutical Chemistry. British Crop Production Council monograph No. 42, pp 131–138
9. **Lee KY, Townsend J, Tepperman J, Black M, Chui CF, Mazur B, Dunsmuir P, Bedbrook J** (1988) The molecular basis of sulfonylurea resistance in tobacco. EMBO J **7:** 1241–1248
10. **Leutwiler LS, Haugh-Evans BR, Meyerowitz EM** (1984) The DNA of *Arabidopsis thaliana*. Mol Gen Genet **194:** 15–23
11. **Maniatis T, Fritsch EF, Sambrook J** (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. **Mazur B, Chui C, Smith JK** (1987) Isolation and characterization of plant genes coding for acetolactate synthase, the target enzyme for two classes of herbicides. Plant Physiol **85:** 1110–1117
13. **Mazur B, Falco SC** (1989) The development of herbicide resistant crops. Annu Rev Plant Physiol Plant Mol Biol **40:** 441–470
14. **Nagao RT, Shaw DM, Eckenrode VK, Meagher RB** (1983) Multigene family of actin related sequences isolated from soybean genomic library. DNA **1:** 1–9
15. **Olszewski NE, Martin FB, Ausubel FM** (1988) Specialized binary vector for plant transformation: expression of the *Arabidopsis thaliana* AHAS gene in *Nicotiana tabacum*. Nucleic Acids Res **16:** 10765–10782
16. **Peoples TR** (1984) Arsenal herbicide (AC 252925): a developmental overview. Proc South Weed Sci Soc **37:** 378
17. **Ray TB** (1984) Site of action of chlorsulfuron: inhibition of valine and isoleucine biosynthesis in plants. Plant Physiol **75:** 827–831
18. **Sanger F, Nicklen S, Coulson AR** (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467
19. **Saari LL, Cotterman JC, Primiani MM** (1990) Mechanism of sulfonylurea herbicide resistance in the broadleaf weed *Kochia scoparia*. Plant Physiol **93:** 55–61
20. **Sathasivan K, Haughn GW, Murai N** (1990) Nucleotide sequence of a mutant acetolactate synthase gene from imidazolinone resistant *Arabidopsis thaliana* var. columbia. Nucleic Acids Res **18:** 2188
21. **Saxena P, King J** (1988) Herbicide resistance in *Datura innoxia*. Cross-resistance of sulfonyl urea resistance lines to imidazolinones. Plant Physiol **86:** 863–867
22. **Saxena PK, King J** (1990) Lack of cross resistance of imidazolinone-resistant cell lines of *Datura innoxia* P. Mill. to chlorsulfuron: evidence for separable sites of action on the target enzyme. Plant Physiol **94:** 1111–1115
23. **Schloss JV, Ciskkanik LM, VanDyk DE** (1988) Origin of the herbicide binding site of acetolactate synthase. Nature **331:** 360–362
24. **Shaner DL, Anderson PC, Stidham MA** (1984) Imidazolinones: potent inhibitors of acetohydroxy acid synthase. Plant Physiol **76:** 545–546
25. **Shaner DL, Anderson PC** (1985) Mechanism of action of the imidazolinones and cell culture selection of tolerant maize. *In* M Zaitlin, P Day, A Hollanender, eds, Biotechnology in Plant Science. Academic Press, New York, pp 287–299
26. **Singh BK, Stidham MA, Shaner DL** (1988) Assay of acetohydroxy acid synthase. Anal Biochem **171:** 173–179
27. **Stalker DM, McBride KE, Malyj LD** (1988) Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. Science **242:** 419–423
28. **Subramanian M, Hung HU, Dias JM, Miner VM, Butler JH, Jachettia JJ** (1990) Properties of mutant acetolactate synthase resistant to triazolopyrimidine sulfonanilide. Plant Physiol **94:** 239–244
29. **Wiersma PA, Schmiemann MG, Condie JA, Crosby WL, Moloney MM** (1989) Isolation, expression and phylogenetic inheritance of an acetolactate synthase gene from *Brassica napus*. Mol Gen Genet **219:** 413–420
30. **Winder T, Spalding M** (1988) Imazaquin and chlorsulfuron resistance and cross resistance in mutants of *Chlamydomonas reinhardtii*. Mol Gen Genet **213:** 394–399